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Talanta 233 (2021) 122568

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Comparison of different digestion methods for proteomic analysis of isolated cells and FFPE tissue samples

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ARTICLE INFO

Keywords:
Proteomics
FASP
Pressure-cycling digestion
In-solution digestion
Cellular subpopulations
FFPE tissue Proteomics

ABSTRACT

Proteomics of human tissues and isolated cellular subpopulations create new opportunities for therapy and monitoring of a patients' treatment in the clinic. Important considerations in such analysis include recovery of adequate amounts of protein for analysis and reproducibility in sample collection. In this study we compared several protocols for proteomic sample preparation: i) filter-aided sample preparation (FASP), ii) in-solution digestion (ISD) and iii) a pressure-assisted digestion (PCT) method. PCT method is known for already a decade [1], however it is not widely used in proteomic research. We assessed protocols for proteome profiling of isolated immune cell subsets and formalin-fixed paraffin embedded (FFPE) tissue samples. Our results show that the ISD method has very good efficiency of protein and peptide identification from the whole proteome, while the FASP method is particularly effective in identification of membrane proteins. Pressure-assisted digestion methods generally provide lower numbers of protein/peptide identifications, but have gained in popularity due to their shorter digestion time making them considerably faster than for ISD or FASP. Furthermore, PCT does not result in substantial sample loss when applied to samples of 50 000 cells. Analysis of FFPE tissues shows comparable results. ISD method similarly yields the highest number of identifications. Furthermore, proteins isolated from FFPE samples show a significant reduction of cleavages at lysine sites due to chemical modifications with formaldehyde-such as methylation (+14 Da) being among the most common. The data we present will be helpful for making decisions about the robust preparation of clinical samples for biomarker discovery and studies on pathomechanisms of various diseases.

1. Introduction

Proteomics data from patient samples are an invaluable source of information in clinical research. However, the quality of results is strongly affected by the technology used for sample preparation. The efficiency and reproducibility of sample preparation in bottom-up proteomics are affected by the quality of cell lysis and protein digestion. Currently, there is no universal method accepted for sample preparation in proteomics. Nevertheless, there have been many studies performed to optimize methods of sample preparation dedicated for particular kinds of biological material; e.g. heart tissue [2] frozen spleen, skin, pancreatic tumor samples [3] or formalin-fixed, paraffin-embedded (FFPE) tissue [4]. The optimal approach for sample preparation should be characterized by robustness and reproducibility of protein extraction,

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https://doi.org/10.1016/j.talanta.2021.122568
Received 3 February 2021; Received in revised form 22 May 2021; Accepted 26 May 2021
Available online 28 May 2021

constant/predictable time of sample processing, and potential for the process automation [5,6]. All these criteria are crucial to efficiently produce reliable results that will elucidate biological questions.

Due to the technical development, which resulted in setting up instrumental platforms that combine high sensitivity with short analysis time, proteomic analyses reach beyond basic scientific research. This advance in proteomics has led to an increasing number of possible clinical applications, such as profiling patient samples [7]. Development of nanoscale liquid chromatography has increased the reliability and ease of use of nanoscale separation methodology. However, there is still a need to streamline and automate these methods making them easier to implement into daily clinical routine [8]. Currently, mass spectrometers (MS) significantly improved its sensitivity, which results, among other factors, from optimization of acquisition rates during LC introduction. Thus, a high number of small samples can be used for analysis and provide robust data. This is of great importance for the analysis of samples of limited availability in a reasonable time scale [9,10].

With increasing knowledge about cell biology and the physiology of organisms, it became clear that high heterogeneity of biological samples [11,12] is important factor that should be considered during the data analysis. Isolation of cellular subsets is a promising approach to improve data credibility and overcome heterogeneity of the biological samples. Fluorescent-activated cell sorting (FACS) [11,12] and immunomagnetic isolation [13] are two of the most common methods used for the separation of cell subsets that have been successfully implemented in proteomic studies. In the case of FFPE samples, isolation of tissue regions containing cells of interest could be done by cutting tissue specimens into thin sections on a microtome and excising them with laser-capture microdissection (LCM) [14]. Using proteomics to better understand the physiology of immune cells and cancer tissue is of great interest nowadays, as our understanding of the cancer-immune cross talk remains limited.

Even though tissue samples provide a more accurate picture of biological pathways, their availability is usually limited, and sample preparation of tissues requires more extensive protocols before they can be used for proteomic analysis. Additionally, in case of FFPE preserved tissues, the additional steps to remove paraffin have to be done before mass spectrometry analysis. Isolated cells from human materials like biopsies are often available only in limited amounts, which drives decisions about how samples are processed due to the available sensitivity of MS detection and expected adsorptive losses during sample preparation from such minimal sample amounts [15]. Moreover, poorly soluble proteins, such as integral membrane proteins, require strong denaturants for extraction and efficient digestion. A harsh denaturation with detergents, chaotropes, or organic solvents [16] is usually necessary to improve isolation and the identification rate of hydrophobic membrane proteins.

To date, there are several reports to compare sample preparation of the cellular subsets [17,18] and FPPE tissue sections [19–21]. A wide variety of methods have been found suitable for processing samples of limited amounts, such as filter-aided sample preparation (FASP) [22], in stage-tip digestion [23] and a pipette tip microreactor-based digestion [18]. Moreover, the most recently published proteomic protocols such as nano-POTS [24,25], single-cell proteomics [26], or lab-on-CHIP [27] suggest that the sample volume will soon be reduced to several or even single cells. However, this substantial improvement in proteomic sample preparation allows single cell analysis to come at the expense of workflow complexity. Low throughput and the complexities of these new methods will likely slow widespread implementation. Here, we have evaluated several sample preparation methods that can routinely be used in most proteomic laboratories that reach down to sub-microgram levels of protein analyzed. In this study, we compared in solution digestion (ISD) and FASP with the recently developed pressure cycling digestion (PCT) technology for sample processing of $CD4^+$ T cells obtained after immunomagnetic isolation from donor blood. Additionally, we evaluated these methods on FFPE tissue sections obtained from human glioblastoma (GBM) tissue.

In-solution digestion is the most classical digestion protocol used in proteomics. Urea is one of most common denaturants used to improve solubility and protease accessibility of proteins. Other denaturants may be used, as long as they are compatible with downstream analysis or can be efficiently removed [28,29]. FASP method was developed to address the known issues of other methods, such as poor coverage of membrane proteins, difficulties in complete solubilization of proteins and incomplete removal of interfering denaturant [30]. It is based on retention of undigested proteins on mass cutoff filter unit, which facilitates efficient denaturant removal and provides semi-solid state digestion conditions. After completion of digestion, peptides can be recovered as they can pass the filter. Over time, the method was improved to provide better robustness [31] or improved sample recovery [32]. Pressure-cycling digestion was developed to enhance trypsin activity and reduce time necessary for complete protein digestion [1,33] as well as facilitate lysis of very resistant samples [34]. It is based on cycling between atmospheric pressure and high pressure (up to 40 kpsi) in the course of lysis and digestion.

In the present paper we test three different methods of the sample preparation: 1) PCT – Methods paragraph 3.3, IV 2)FASP – Methods paragraph 3.4, 3) ISD – Methods paragraph 3.5 for analysis of whole proteome and membrane proteome of pure sorted cell subsets ($CD4^+$ T cells) and 15–20 μm thick FFPE tissue slices. Thus, the paper provides a comprehensive overview of how the recently developed PCT proteomic sample preparation can be applied for analysis of two distinct biological materials compared to the widely used and well-established FASP and ISD proteomic sample preparation methods.

2. Materials

Acetonitrile (ACN), ethanol, water and formic acid were bought from VWR (Avantor) (PA, USA). Trypsin was obtained from Promega (MA, USA), Lys-C protease was obtained from New England Biolabs (MA, USA), NaCl was obtained from Merck (Darmstadt, Germany) and Tris was obtained from Bioshop. All other chemicals were bought from Sigma (MO, USA).

3. Methods

3.1. Isolation of $CD4^+$ T cells

$CD4^+$ T cells were isolated from buffy coats obtained from volunteer blood donors (Regional Centre for Blood Donation and Treatment in Gdansk, Gdansk, Poland) as described before [35]. Briefly, peripheral blood mononuclear cells (PBMC) were separated with Ficoll/Uropoline gradient centrifugation and then subjected for negative immunomagnetic selection of $CD4^+$ T cells (Cat # 19 052, StemCell Technologies) according to the manufacturer instructions.

3.2. Preparation of FFPE tissue for proteomics

GBM samples were collected and used according to the local ethical approvals (University of Edinburgh, Edinburgh Cancer Research Centre, reference number 06/S1101/16). The study was conducted in accordance with guidelines of the Commission and Declaration of Helsinki. GBM 15 μm and 20 μm thick FFPE tissue sections from a single patient were deparaffinized and rehydrated by a modified protocol inspired by Espina et al. [36]. FFPE tissue sections were first washed for 2 min with xylene to remove paraffin. Deparaffinized GBM tissue was rehydrated for 2 min using a series of graded ethanol washes (100% EtOH, 85% EtOH, 70% EtOH and deionized water). Subsequently, each GBM tissue slide was cut into three sub-sections of approximately 16 mm^2, which were then scraped and transferred into Teflon PCT tubes. Therefore, method performance was evaluated on independent but adjacent tumor subsections. To address the sensitivity of our assay, a peptide digest

corresponding approximately to one half of a tissue subsection was used for each LC-MS run.

3.3. Pressure-assisted lysis and digestion (PCT and PCT/ACN)

For PCT lysis and digestion we used modification of protocol described in Gao et al. [37]. $CD4^+$ cell pellets (1×10^6 cells) were resuspended in 40 μl of buffer (4 M urea/50 mM NH_4HCO_3/10 mM Tris (2-carboxyethyl)phosphine (TCEP)/40 mM iodoacetamide (IAA) or 4 M urea/50 mM NH_4HCO_3/10 mM TCEP/40 mM IAA/30% ACN) and placed into PCT microtube. Cell lysis was done in Barocycler 2320EXT (Pressure Biosciences, MA, USA) by performing 45 pressure cycles (50 s at 30 kpsi/10 s at low pressure) at 35 °C. Subsequently, 0.7 μg of Lys-C protease was added, and samples were digested using 99 cycles (50 s at 20 kpsi/10 s at low pressure) at 35 °C. Then, samples were diluted by addition of 100 μl of 50 mM NH_4HCO_3 and 1 μg of trypsin (Promega, MA, USA) was added into the samples. Samples were digested using 99 cycles (50 s at 20 kpsi/10 s at low pressure) at 35 °C. Digestion was quenched with 650 μl of 0.2% TFA and PCT tubes were washed thoroughly with 0.2% trifluoroacetic acid (TFA) to collect all remaining sample.

3.4. FFPE tissue PCT lysis and digestion

FFPE tissue lysis and protease digestion was performed using a protocol inspired by Zhu et al. [38]. Briefly, GBM 20 μm and 15 μm thick FFPE tissue sections tissue were scrapped into 100 μl of 100 mM NH_4HCO_3/30% ACN lysis buffer and vortexed. Further, samples were transferred into clean PCT microtubes (Pressure Biosciences, MA, USA) and closed with 100 μl microcap. Tissue lysis was performed with 60 pressure cycles (20 s at 32 kpsi/15 s at low pressure) at 80 °C in Barocycler 2320EXT. Lysates were subjected to ISD or FASP digestion, or further processed for PCT digestion, as follows. Dithiotreitol (DTT) was added to final concentration of 20 mM into the lysates and samples were incubated at 37 °C for 30 min. Alkylation reaction was performed by adding IAA to a final concentration of 60 mM and samples were incubated in a dark at room temperature for 30 min. Subsequently, digestion was performed by addition of 20 μl of 5 ng/μl trypsin (Promega, MA, USA) in 100 mM NH_4HCO_3. Proteins were digested using 60 pressure cycles (50 s at 25 kpsi/10s at low pressure) at 37 °C in Barocycler 2320EXT. Digestion was quenched by addition of 50 μl of 5% TFA.

3.5. In-solution digestion (ISD)

In-solution digestion od $CD4^+$ cells and FFPE tissue was performed by a protocol inspired by Medzihradszky et al. [39] and Dapic et al. [40]. Briefly, $CD4^+$ T cell pellets (1×10^6 cells) were resuspended in 100 μl of ISD lysis buffer (8 M urea/30% ACN/100 mM NH_4HCO_3) and samples were incubated at 37 °C for 30 min with shaking. Reduction of disulfide bonds was achieved by addition of DTT to final concentration of 20 mM and incubation for 30 min at 37 °C with shaking. Then, IAA was added at final concentration of 60 mM, and samples were incubated in dark for 30 min at 37 °C. Subsequently, samples were diluted with 880 μl of water and 120 μl of 1 M NH_4HCO_3. For digestion, 2 μg of trypsin (Promega, MA, USA) was added, and samples were digested for 18 h at 37 °C. Digestion was quenched by addition of 50 μl of 5% TFA and samples were diluted to 1.5 ml with 0.1% TFA.

100 μl of FFPE lysates from both 20 μm and 15 μm thick tissue sections were reduced by adding DTT up to 20 mM final concentration at 37 °C for 30 min. Alkylation was performed by addition of IAA to final concentration of 60 mM and samples were incubated in dark at room temperature for 30 min 20 μl of 5 ng/μl trypsin (Promega, MA, USA) in 100 mM NH_4HCO_3 was added to the protein extract and samples were digested overnight at 37 °C. Digestion was quenched with 50 μl of 5% TFA.

3.6. Filter-aided sample preparation (FASP)

Filter aided sample preparation of $CD4^+$ cells and FFPE tissue was performed according to a protocol inspired by Wisniewski et al. [30]. Briefly, $CD4^+$ T cell pellets (1×10^6 cells) were resuspended in 40 μl of FASP lysis buffer (1% sodium dodecyl sulfate (SDS)/0.1 M Tris at pH 7.6) and incubated for 10 min at 95 °C. Next, the samples were cooled down to 37 °C and incubated with 0.1 M DTT for 30 min. Then 200 μl of urea solution (8 M urea/0.1 M Tris pH 8.5) was added, and samples were sonicated for 20 min at room temperature, at 100% power in Emmi D60 (Emag, Germany) ultrasonic bath. Debris were removed by centrifugation at 14 000 g for 15 min and samples were transferred to Microcon 10 kDa cutoff filters (Merck, NJ, USA). Samples were further centrifuged for 15 min and washed with 200 μl of urea solution. Then, 100 μl of 50 mM IAA in urea solution was added, and samples were incubated for 20 min in dark at room temperature. After centrifugation for 15 min, filters were additionally washed three times with 200 μl of urea solution, and three times with 100 μl of 50 mM NH_4HCO_3. Subsequently, 2 μg of trypsin in 40 μl of 50 mM NH_4HCO_3 was added, and samples were incubated at 37 °C overnight. After digestion, peptides were recovered by centrifugation, and washing filters with 50 μl of 0.5 M NaCl. Samples were diluted to 1 ml with 0.2% TFA.

Lysates of 20 μm thick FFPE sections (80 μl) were diluted in 100 μl of 8 M urea/0.1 M Tris/HCl at pH 8.5 (urea solution) and added to Vivacon 500 10 kDa cut-off filter (Sartorius Stedim Biotech, Germany). Samples were centrifuged at 15 000 g/20 min to pass all liquid through the filter unit. Proteins were reduced by adding 100 μl of 16.7 mM TCEP in urea buffer on a thermomixer at 37 °C for 60 min at 600 rpm, followed by centrifugation at room temperature at 15 000 g until all liquid passed through the filter unit. Then, 100 μl of 300 mM IAA in urea solution was applied to samples, and samples were incubated for 20 min in dark at room temperature. Unreacted alkylating reagent was filtered through the filter unit by centrifugation at room temperature at 15 000 g. Three washes using 100 mM NH_4HCO_3 were performed to diminish urea from filter unit prior proteolytic digestion. Subsequently, 1 μg of trypsin (Promega, MA, USA) in 100 μl of 50 mM NH_4HCO_3 was added and samples were incubated at 37 °C overnight. Peptides were recovered from the filter by centrifugation at 15 000*g* for 20 min.

3.7. Peptide desalting prior to liquid chromatography/mass spectrometry (LC/MS) analysis

Samples of $CD4^+$ T cell derived peptides were diluted with 0.2% TFA to reach 1 ml of the sample volume and loaded on Supelclean 100 mg columns (Supelco, PA, USA). After loading, peptides were washed with 0.05% TFA, and desalted peptides were eluted by 80% ACN in 0.05 TFA/H_2O. Eluates were dried using a SpeedVac concentrator (Thermo Scientific, MA, USA) and stored at −80 °C until instrumental analysis.

Peptides obtained from FFPE tissue were loaded on [41]MicroSpin C18 Columns (Harvard Apparatus, MA, USA) and were desalted using 0.1% formic acid (FA). Desalted peptides were then sequentially eluted using 50% ACN followed by 80% ACN in 0.1% FA/H_2O and finally with 0.1% FA in ACN. Peptide eluates were pooled and dried using a SpeedVac concentrator (Thermo Scientific, MA, USA) and stored at −80 °C until instrumental analysis.

3.8. LC/MS analysis

3.8.1. $CD4^+$ T cell subsets

Three independent samples were prepared for each analysis. Samples of peptides isolated from $CD4^+$ T cells were analyzed on RSLCnano 3000 nanoLC system (Thermo Scientific, MA, USA) coupled to LTQ XL Orbitrap mass spectrometer (Thermo Scientific MA, USA) by Advion ChipMate nanoESI source (Advion, NY, USA). Ion source capillary temperature was set to 150 °C and spray voltage to 1.8 kV. Prior to LC-MS, samples containing peptides from $CD4^+$ T cells were dissolved in 40

μl of loading buffer (0.08% TFA/2% ACN) and 6 μl were loaded onto a 300 μm × 5 mm PepMap100 cartridge trap column (Thermo Scientific MA, USA) at flow rate of 5 μl/min for 10 min. Samples were further submitted to 150 mm × 75 μm PepMap RSLCnano column (Thermo Scientific MA, USA). Mobile phase A was composed of 0.1% FA in water and mobile phase B of 80% ACN/0.08% FA. Gradient separation were conducted as follows: 2.5% B for 10 min, then 120 min gradient to 50% B, 2 min gradient to 99% B, 5 min at 99% B, 2 min gradient back to 2.5% B, and 2.5% B for 11 min (150 min total analysis time). Data were acquired in DDA mode, MS spectra in range of 300–1600 *m/z* were collected at resolution of 30 000 in Orbitrap analyzer with maximum injection time of 200 ms and top 10 precursors of minimum intensity of 3000 were fragmented using normalized collision energy of 35% with activation Q set to 0.25 and activation time to 60 ms. MS/MS spectra were acquired using ion trap with maximum injection time of 100 ms and automatically adjusted *m/z* range. Scan rate was set to Normal.

3.8.2. GBM tissue sections

FFPE tissue peptide containing samples were dissolved in 25 μl of 0.05% TFA in 5% ACN prior to LC-MS. 10 μl of each peptide sample corresponding approximately to 0.5 μg of protein (estimated from NanoDrop 2000 (Thermo Scientific, MA, USA) absorbance at 280 nm) were analyzed in single technical replicate on an Eksigent Ekspert nanoLC 400 (SCIEX, Canada) coupled to TripleTOF 5600+ (SCIEX, Canada) mass spectrometer. Peptides were loaded on a trap column (300 μm i. d. × 5 mm) packed with C18 PepMap100 sorbent of 5 μm particle size (Thermo Scientific MA, USA). Further, peptides were washed for 10 min using 0.05% TFA in 5% ACN. An analytical gradient of ACN/water (flowrate 300 nl/min) was used to elute the peptides on an analytical capillary emitter PicoFrit® nanospray column (75 μm i. d. × 210 mm (New Objective), MA, USA) self-packed with ProntoSIL 120-3-C18 AQ sorbent with 3 μm particles (Bischoff, Germany). Analytical gradient was composed from mobile phase A (0.1% (v/v) FA), and mobile phase B (0.1% (v/v) FA in ACN). Column was first equilibrated for 30 min at 5% B. Gradient peptide separation started at 5% B and linearly increased up to 40% B in following 120 min. Column wash was performed by increasing mobile phase B from 40% up to 80% in 5 min and subsequently keeping this mobile phase composition for 5 min. The proportion of mobile phase B linearly decreased from 80% to 5% in 2 min after column wash. Column equilibration was held for 28 min at 5% B. Output from the separation column was coupled to an NANOSpray® III ion source (SCIEX, Canada). Nitrogen was the drying and nebulizing gas. The heater temperature was set to 150 °C and the voltage at the capillary was 2.65 kV. Data were acquired in data-dependent analysis (DDA) mode. MS1 mass range was restricted from *m/z* 400 up to *m/z* 1250 and MS2 range was restricted from *m/z* 200 up to *m/z* 1600. Each data-dependent cycle included collecting of MS2 spectra from 20 the most intensive precursor ions. Each MS2 spectrum was collected for 150 ms resulting in a 4.8 s method cycle time. Once measured precursor ions were excluded for 19 s. Precursor ions with intensity lower than 200 counts per second (cps) were excluded. The extraction of mass spectra from raw data was performed using Protein Pilot 4.5 (SCIEX, Canada).

3.9. Data analysis

Qualitative data analyses were done by X!Tandem search engine embedded within PeptideShaker/SearchGUI software suite [42] For $CD4^+$ T cells, mgf input files were generated from raw data using MSConvert [43]. For FFPE data, mgf files were created by ProteinPilot (SCIEX, Canada). All data were recalibrated before analysis – in case of $CD4^+$ T cells, it was done by PeptideShaker [44], and for FFPE, data were recalibrated with ProteinPilot. Searches were done against human Uniprot human reference proteome database (downloaded on December 25, 2020) with concatenated reverse decoy database. For X!Tandem main search cysteine carbamidomethylation was considered as fixed modification, and methionine oxidation as a variable modification. "Quick Acetyl" for N-term acetylation, and "Quick Pyrolidone" for N-terminal Q modification checks were applied. For model refinement (second-stage X!Tandem search) deamidation of asparagine and glutamine were additionally considered. For $CD4^+$ T cell data, acquired on LTQ Orbitrap, mass tolerances were 10 ppm for parent ions, and 0.5 Da for fragment ions. For FFPE data, acquired on TripleTOF 5600+, values were respectively 17 ppm and 0.025 Da.

Semitryptic peptide search was done by X!Tandem with parameters similar as above, but with semi-specific enzyme activity allowed in main search. Furthermore, peptides showing only loss of initial methionine were not counted as a result of semi-specific digestion.

Quantitative data analysis for $CD4^+$ cells data was done using MaxQuant 1.6.12 [45]. For identification, precursor and fragment mass tolerances were set to 4.5 ppm and 0.5 Da. Methionine oxidation was set as variable modification. To enable comparison of relative amounts of material, no normalization was performed.

A search for FFPE tissue modifications was done using $MSGF^+$ (release 2020.03.14) [46] against Uniprot human reference proteome database (downloaded on December 25, 2020) with concatenated reverse decoy database. Carbamidomethylation was set as fixed modification, and lysine methylation +14 Da, K +12 Da, K +30 Da and K +58 Da were considered as variable modifications. Precursor ion mass tolerance was set to 30 ppm and to 30 ppm in MS/MS.

In all cases, 1% false discovery ratio (FDR) was allowed at peptide and protein level.

4. Results and discussion

There are numerous reports of optimization of sample preparation in proteomics, yet there is very little known about comparison of PCT sample homogenization and digestion with the more widely used ISD and FASP methods. Here, we compared PCT, in-solution and FASP-based protocols in terms of overall efficiency, reproducibility, digestion specificity, miscleavage rate and the ability of each to detect membrane proteins.

4.1. Assessment of efficiency of sample preparation methods

To evaluate efficiency of sample preparation protocols for $CD4^+$ cells, samples were compared according to the summed intensity of all peptides identified by MaxQuant software, which we further refer to as peptide amount. The rationale for choosing this metric is that such a value combines the amount of extracted peptide material and efficiency of peptide-spectral match (PSM) identifications. Additionally, we used typical method benchmarking parameters such as number of identifications at the protein and peptide level to compare efficiency of the methods on $CD4^+$ cells and GBM FFPE tissue. These two very different sample types represent both the easily lysed cell suspension and fixed tissue, which is difficult to disrupt and solubilize. We detected clear trends between tested methods, with the most efficient method (ISD) generating nearly seven times more identified peptides compared to PCT-ACN. Results also showed a high consistency in trend between methods in the identified number of proteins and peptides from both $CD4^+$ cells and FFPE GBM tissue (Fig. 2). In both cases, ISD method gave the best overall results.

Results also demonstrated that the amount and number of identified peptides and proteins was lower with PCT compared to ISD – both for cells and FFPE tissue samples (Fig. 1). To check if relatively short digestion time in PCT method was sufficient for complete digestion, we investigated the influence of digestion time on the efficiency of the PCT method (Fig. 3, Supplementary Figure 4). However, prolonging the time of trypsin digestion did not affect the number of identified proteins and peptides, or amount of identified peptides. We have also investigated the effect of addition of ACN, as its use was shown to improve the digestion efficiency in case of in-solution digestion [40,47], possibly by improving the trypsin activity. However, addition of ACN to PCT lysis buffer for

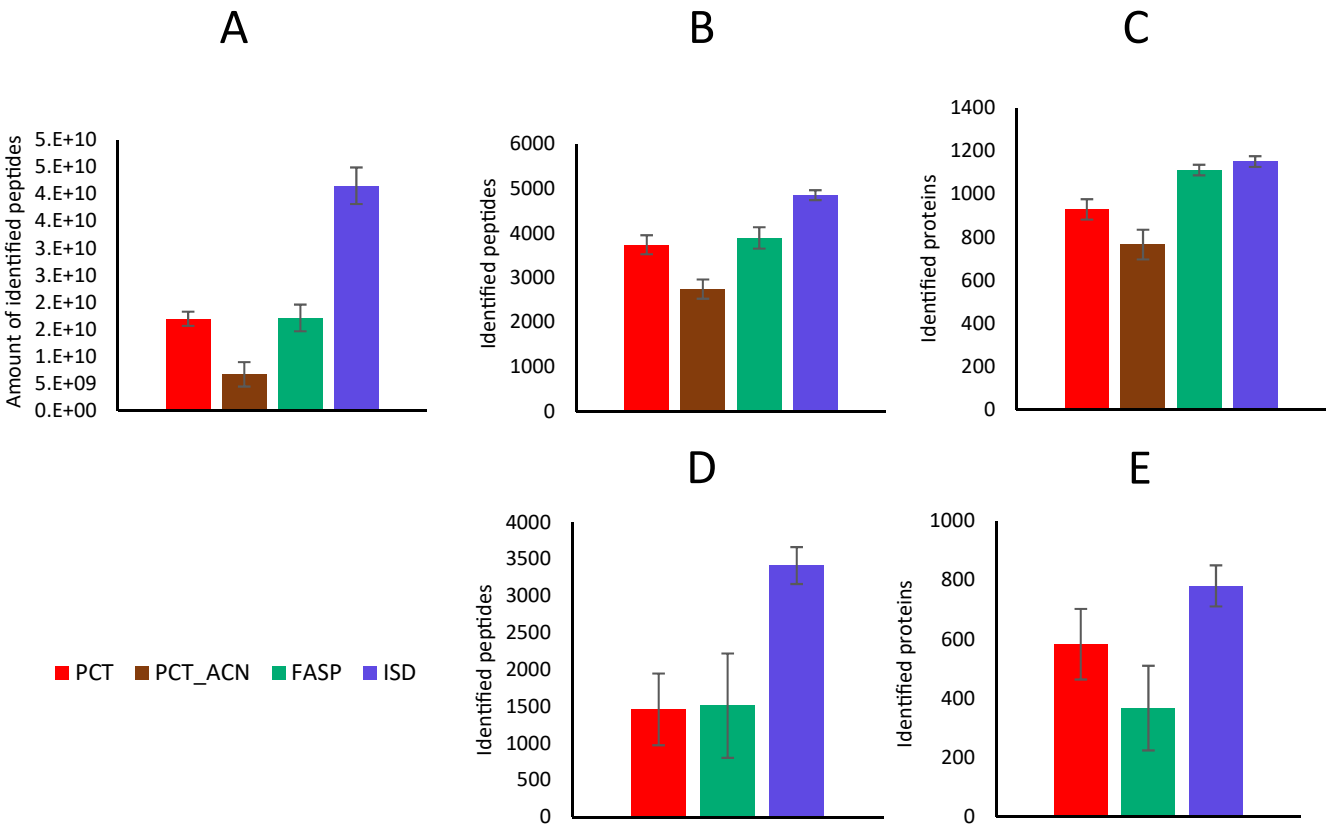

Fig. 1. Assessment of efficiency of different protocols applied on CD4$^+$ cells and FFPE tissue samples. Results for CD4$^+$ cells are given in the panels A-C. *A) Sum of intensities of all identified peptides. B) Number of identified peptides. C) Number of identified proteins. Results are shown as mean ± standard deviation (SD) of three independent samples.* **Results for glioblastoma FFPE tissue are given in panels D and E:** *D) Number of identified peptides. E) Number of identified proteins. Results are shown as mean ± standard deviation (SD) of three independent tissue sections from one glass slide.*

analysis of CD4$^+$ cells led to substantial decrease of the number and amount of identified peptides and proteins.

Interestingly, although the FASP method generated a lower amount of peptides and fewer peptide identifications compared to the ISD method, the number of protein identifications was nearly as high as in ISD. This suggests that the FASP method is effective for digestion of a wide range of proteins that may be resistant to digestion by other methods, which may be related to the initial use of harsh denaturing conditions for solubilization of proteins resistant to denaturation [48]. Obtained results do not support the superiority (in terms of identification efficiency) of PCT digestion over other existing methods. There is shortage of publications comparing PCT with other digestion methods. Several available reports used isolated proteins or protein subpopulations [33] or used PCT only for protein extraction, and subsequent digestion was carried out at ambient pressure using other methods [49]. PCT was shown to be superior in protein extraction efficiency and throughput from FFPE tissue slices [50]. In this work, authors actually compared pressurized protein extraction vs ambient pressure extraction, so there is a possibility that this method is particularly well-suited for lysis of resistant samples, while subsequent digestion do not benefit from increased pressure. This issue would be worth further investigation. There is also a chance that digestion can be further improved by employing MS-compatible detergents [51]. The PCT method has the advantage of being much shorter, with sample preparation time of 5–6 h, compared to 18 h for ISD and FASP methods. Nevertheless, there are also many pressure-free methods for fast sample digestion, mostly available as commercial kits [52,53].

Our results also demonstrated that the sum of the intensities of identified peptides is a sensitive measure that can be used to determine digestion efficiency. It may be particularly useful in cases where traditional means of measuring peptide concentration, such as UV absorption are inaccessible or their sensitivity is too low. Furthermore, it allows to partially differentiate between sample contents and common laboratory contaminants, such as keratins.

4.2. Method reproducibility

To compare reproducibility between the methods, firstly we compared the number of protein groups consecutively quantified in all three runs (Fig. 4A). Most protein groups were quantified without missing values in samples processed with the ISD method (676), and the least in PCT/ACN samples (414). The number of quantified protein groups may be highly dependent on overall signal intensity, as detection of low-abundant ions is hampered with low sample amount – protein groups of low intensity were more often not present in all of the replicates (data not shown). To evaluate the quantitative reproducibility, we compared the distributions of relative standard deviations of measured abundances of quantified proteins. ISD and PCT methods showed slightly higher reproducibility (93% and 96% of protein groups had relative SD < 0.2, respectively) compared to PCT/ACN and FASP methods (87% and 91%, respectively) (Fig. 4B).

Venn diagrams showing reproducibility between individual samples (Supplementary Figure 5) show that in case of CD4$^+$ cells, the overall reproducibility is very good, as can be expected in case of homogenous cultured cells. In case of FFPE tissue slices the reproducibility is much poorer and it could be addressed to tissue heterogeneity originating from sample preparations performed on independent sub-sections from a single FFPE GBM tumor tissue. This is very well known an unavoidable phenomenon originating from tumor heterogeneity. To lessen this effect it would be necessary to employ limited sample preparation methods

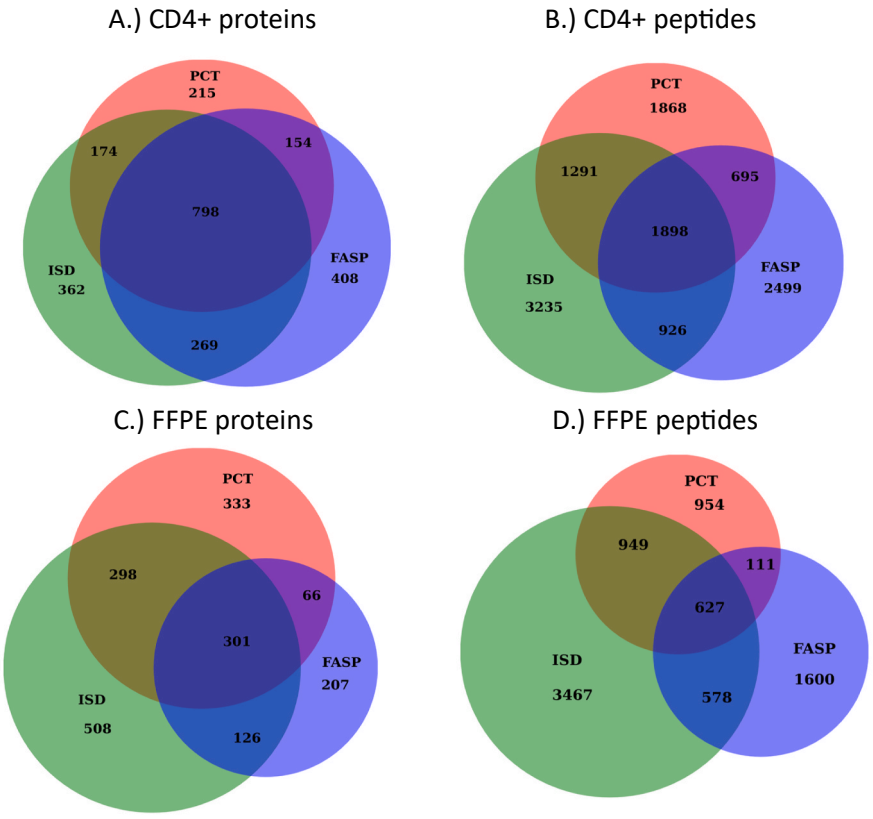

Fig. 2. Venn diagrams showing overlap of identified proteins and peptides in all methods. *As expected, FFPE tissue displays higher variability in terms of peptides and proteins identified compared to CD4$^+$ cells. This variability could be addressed to tissue heterogeneity originating from sample preparations performed on independent sub-sections from a single FFPE GBM tumor tissue.*

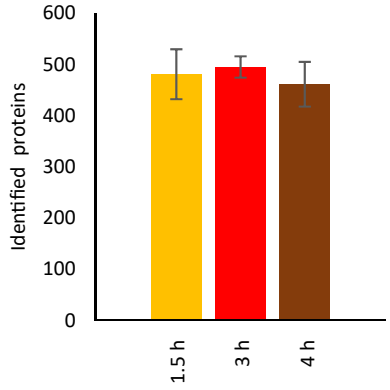

Fig. 3. Effect of digestion time on efficiency of PCT method. After lysis and initial 99 min digestion with Lys-C, samples were digested with trypsin for the indicated amount of time (1.5 h, 3 h or 4 h). Results are shown as mean ± SD of three independent samples.

such as NanoPots in combination with tissue voxelization and supervised selection of homogenous voxels [54]. However, nowadays these methods are still not mature enough to deal with FFPE tissue specimens and still does not fully resolve tissue heterogeneity problem.

4.3. Digestion specificity and missed cleavages

Data showed that specificity of digestion varies considerably between the methods (Fig. 5B,D). PCT/ACN and ISD methods are characterized by high digestion specificity (1% and 2.4% of semitryptic peptides, respectively), while conventional PCT and FASP methods generated a higher amount of semitryptic peptides (5.3% and 8.7%) in CD4$^+$ cell samples. We observed a similar trend in FFPE tissue, but with slightly higher semitryptic peptide rate. In analysis of FFPE GBM tissue ISD showed the highest specificity (2.6% semitryptic peptides) followed by PCT and FASP (6.8% and 10.8% semitryptic peptides, respectively). Furthermore, miscleavage rate analysis revealed that miscleavages might be sample and method dependent (Fig. 5A,C). When using the FASP method a lower percentage of miscleaved peptides (6% in CD4$^+$ cells and 29% in FFPE tissue) was found than in the other methods that generated between 22% and 33% in CD4$^+$ cells or 45% and 47% of miscleaved peptides in FFPE tissue, for ISD and PCT respectively. We have also shown that a lower amount of miscleaved peptides is not an effect of retaining longer peptides on a filter membrane (Supplementary Figure 3). These results indicate that digestion characteristics vary between the different methods used, possibly indicating different conditions during digestion. In a case of FASP, the miscleavage rate is much lower and simultaneously, the digestion specificity is reduced in both CD4$^+$ and FFPE tissue. It suggests much higher trypsin activity in semi solid-phase and denaturant-free conditions in FASP. Lower miscleavage rate was previously reported in Refs. [2,55], however the digestion specificity is not discussed in these papers.

Generally, Fig. 5A and C shows characteristic increase in miscleavage rate in FFPE tissue compared to CD4$^+$ cells regardless of the digestion method used. This observation could be explained by the frequent modification of lysine residues as a result of formaldehyde treatment in FFPE tissue. This was further analyzed by K/R parameter at the C-term of tryptic peptides yielded from studied FFPE tissue. Supplementary Figure 1 shows that K/R is lower in FFPE tissue compared to non-fixed CD4$^+$ cells. This is in agreement with previously published work by Sprung et al. and Broeckx et al. [56,57] where the authors demonstrated a characteristically decreased preference of lysine cleavage sites

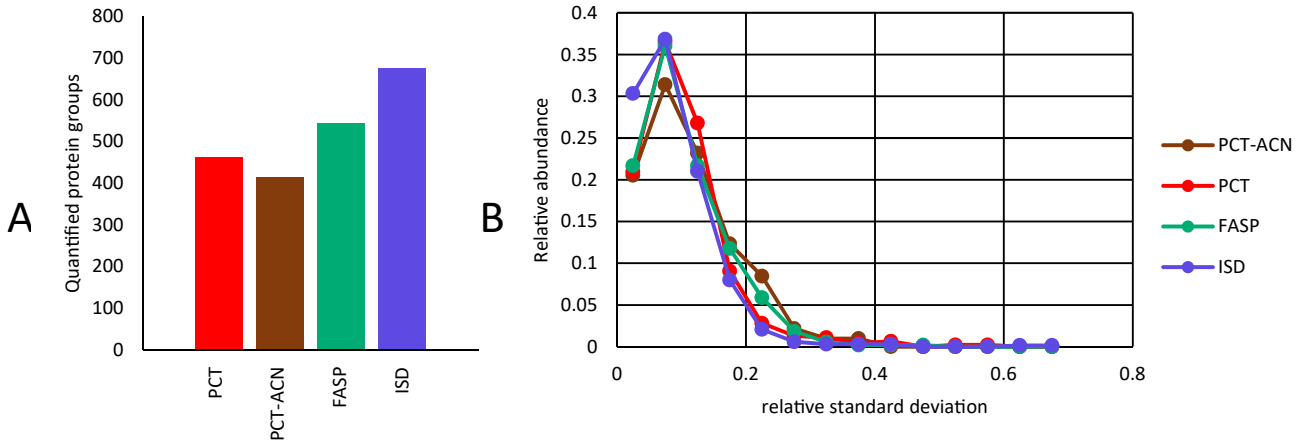

Fig. 4. Evaluation of reproducibility of different sample preparation methods used for CD4$^+$ cells. A) total numbers of protein groups quantified consecutively in three independent samples by MaxQuant software. B) distributions of relative standard deviations of LFQ intensity for proteins groups quantified in three independent samples.

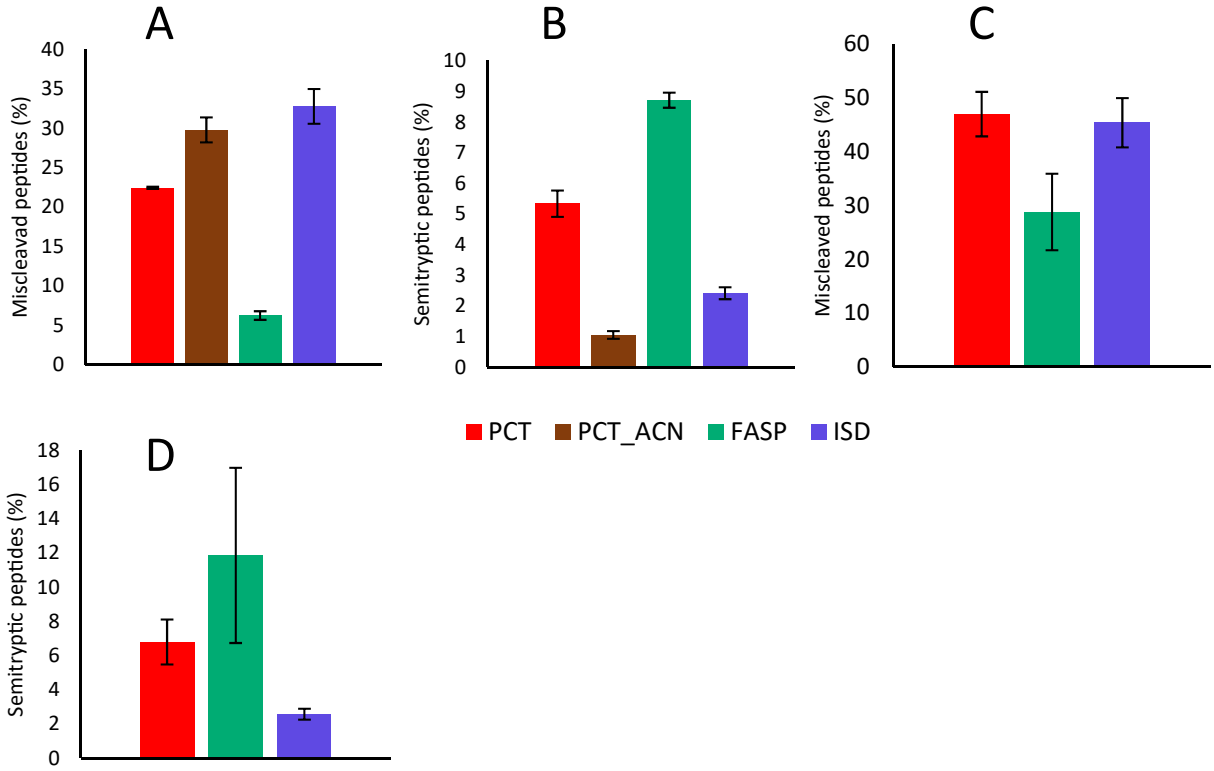

Fig. 5. Comparison of digestion specificity and missed cleavages for used digestion methods. *A) Miscleaved peptides in CD4$^+$ cells samples digested with different methods. B) Semitryptic peptides in CD4$^+$ cells samples digested with different methods. C) Miscleaved peptides in FFPE tissue samples digested with different methods. D) Semitryptic peptides in FFPE tissue samples digested with different methods. Results are shown as mean ± SD) of three independent tissue sections from one glass slide or three independent cell samples.*

compared to arginine cleavage sites in FFPE biological material. Therefore, next we focused on lysine side chain modifications in FFPE tissue.

4.4. Analysis of FFPE tissue related modifications

It is well known that FFPE tissues undergo protein modification and crosslinking during formaldehyde treatment. Formaldehyde reacts with primary amines such as lysine side chains forming reactive hydroxymethyl-methylol groups (+30 Da) which could further react with primary amides and secondary amines forming methylene bridges (+12 Da). Methylol groups could further undergo water loss and form a Schiff's base (+12 Da). Additionally, several other modifications such as an attachment of two methylol groups (+60 Da), dimethylation (+28 Da) could be found [21,58]. Despite quite extensive knowledge about formaldehyde reactions with proteins, observed modification patterns on peptides derived from FFPE tissues are not well-defined. Therefore, we focused on identification of known FFPE related modifications on lysine side chains which were previously reported [21,59]. Fig. 6A compares average normalized number of FFPE related modifications, which were confidently identified in three independent FFPE tissue sections from one glass slide compared across sample preparation

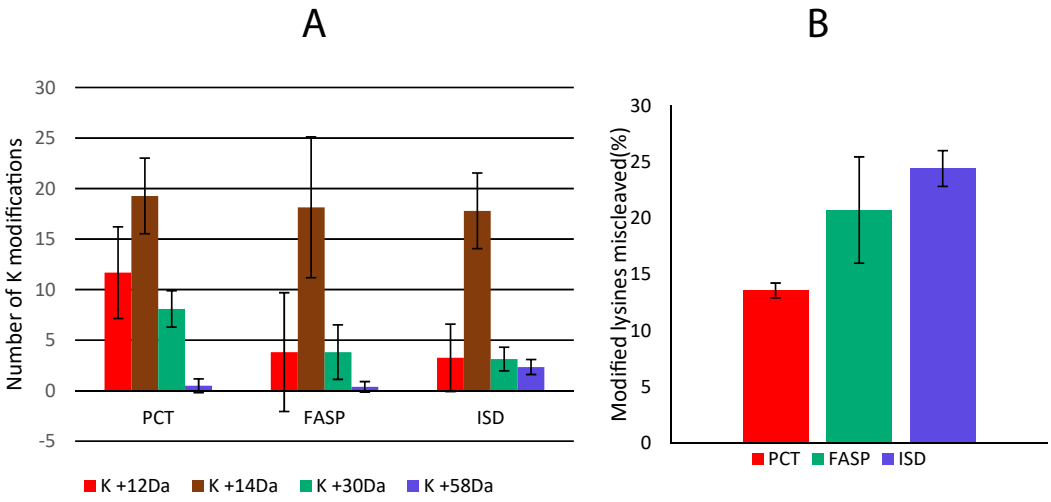

Fig. 6. Lysine modifications in FFPE tissues *A) Identified FFPE related lysine side chain modifications in GBM FFPE tissue between compared sample preparation methods, each technique performed on three independent FFPE tissue sections from one glass slide B) Contribution of modified lysine residues to overall miscleavage rate on lysine in FFPE tissue. Results from three samples show slight contribution (up to 20%) of modified lysine to overall miscleavage rate on lysine across compared sample preparation methods.*

workflows. We observed a significant increase of +14 Da modification on lysine side chains across all sample preparation workflows reflecting methylation introduced by a reaction of primary amino group with formaldehyde. An increase in +14 Da modification on side chain of lysines was observed as characteristic for FFPE tissue compared to non-fixed biological material (Supplementary Figure 2B). Recent work from Tabb et al. ranked the frequency of FFPE related modification identification based on various database search approaches. Overall, as the most abundant modification authors identified +14 Da modification on lysine and N-term referring to methylation. On the other hand, +30 Da lysine modification in Tabb et al. dataset fell out TOP 10 PTMs probably due to wider range of investigated modifications [60].

Moreover, our data are in agreement with previously published work by Zhang et al. and Coscia et al. who reported increased on lysine methylation in FFPE tissue in terms of spectral counts and XIC-based label free quantitation [59,61].

In Supplementary Figure 1B we demonstrated that decreased K/R at C-terms of peptides and increased miscleavage rate (Fig. 5C) is characteristic for FFPE tissue. Therefore, next we focused on lysine miscleaved peptides to investigate whether lysine side chain modifications contribute to increased miscleavage rate. Fig. 6B shows a miscleavage rate related to modified lysine side chains as a percentage of all lysine related miscleavages. Fig. 6B shows that modified lysine slightly contributes to elevated miscleavage rate characteristic in FFPE tissue. We also observed that trypsin cleavage will only rarely occur at modified lysine as almost no modified lysines were identified at the C-terminal position. Toews et al. showed that the extent of FFPE characteristic protein modifications in FFPE tissue is dependent on the overall time of formaldehyde treatment and formaldehyde concentration used for treatment [62]. Therefore, FFPE tissue fixation protocol might have an impact on tryptic miscleavage rate during proteomic sample preparation. As expected, methylation of lysine side chains contributes mostly to the modification related tryptic miscleavage (Supplementary Figure 2A). Taken together, we recommend setting at least methylation on lysine (K+14 Da) in variable modification list in FFPE proteomic dataset analyses.

4.5. Identification of integral membrane proteins

Data analysis of integral membrane protein (IMP) retrieval showed that the FASP procedure was the most efficient in membrane protein identification due to harsh denaturing conditions (Fig. 7). From all detected proteins, 7.5% in $CD4^+$ cells were integral membrane proteins.

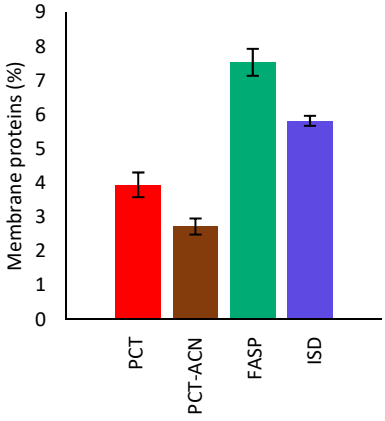

Fig. 7. Identified integral membrane proteins between methods. *Integral membrane proteins were annotated using Gene Ontology annotations (GO term GO:0016021 "integral component of membrane" and its children terms) . Results are shown as mean ± SD) of three independent samples.*

This is also in agreement with overall higher protein diversity in FASP-digested proteome. The contribution of integral proteins in the ISD method was 5.9% despite the lack of detergent-based denaturation. Both PCT and PCT/ACN methods showed a lower proportion of IMPs (4% and 2.7%, respectively).

Furthermore, the integral membrane protein proportion in the database was 17.5%, while there is substantial number of poorly annotated proteins of unreviewed sequences. Therefore, experimentally detected values are applicable for relative comparison between methods, however actual percent of IMPs may be higher due to high proportion of hits for non-reviewed sequences in the search results.

4.6. Assessment of sensitivity with limited amounts of starting material

To assess the sample losses during the PCT method, we have compared efficiency for sample preparation from 50 k, 100 k, 200 k and 500 k $CD4^+$ cells (Fig. 8). To directly compare the amount of material loss, the same theoretical amount of material (corresponding to 5 k cells) was analyzed. Possible contaminant proteins were excluded, which might be important for lower starting material amount whereas contaminant-derived peptides may contribute to substantial percentage of signal intensity. While the number of identified proteins and peptides

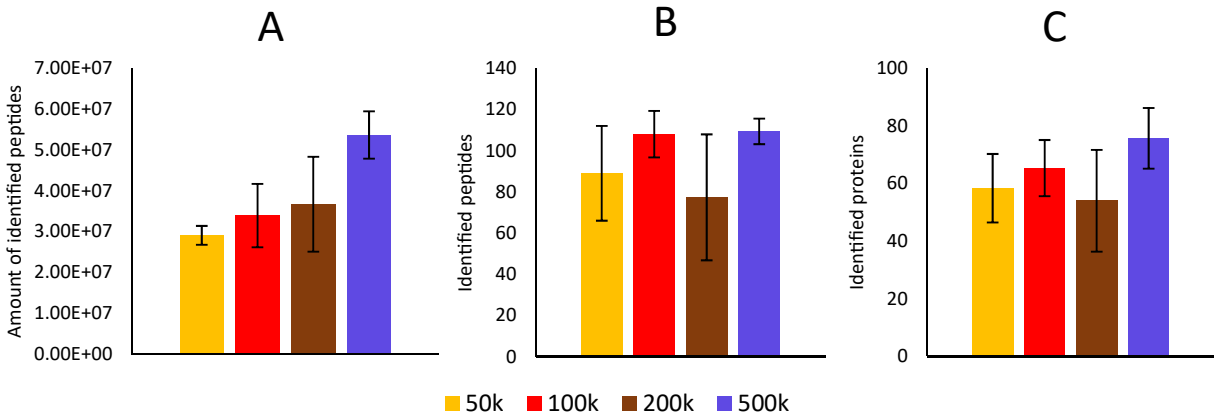

Fig. 8. Efficiency of PCT method for sample preparation of 50k, 100k, 200k and 500k cells. *5000 cells were analyzed from each sample. A) Sum of intensities of all identified peptides. B) Number of identified peptides. C) Number of identified proteins. Results are shown as mean ± SD of three independent samples.*

are not substantially affected by reducing the amount of starting material, the amount of identified peptides shown as sum of intensities of precursors was lower in 50 k, 100 k and 200 k samples. These results demonstrated the application of standard PCT method for sample preparation of samples with 50 000–100 000 cells.

5. Conclusion

We compared pressure-assisted sample preparation method with the classical in-solution digestion and FASP method on $CD4^+$ T cells isolated from blood and FFPE GBM tissue slices. These data come from two different laboratories, and the analyses were done completely independently on two LC-MS platforms. This fact could serve as additional confirmation of the validity of the observed differences.

Our data demonstrates that the ISD method, which requires longer digestion time but very little hands-on time, could be used as low-cost and low-complexity alternative to the two other methods. This method is also characterized by high robustness and can be performed without risk of sample loss even by researcher without experience in proteomic sample preparation. FASP has the best performance for analysis of membrane proteins and shows clearly different pattern of trypsin activity, reducing miscleavage rate but also digestion specificity. Unfortunately, there are no literature describing details of digestion process on filter. The main advantages of PCT are short sample preparation time (however this is not unique to this method, as faster and easier methods exist) and ability do lyse very resistant samples, however there is a need to determine the optimal conditions for pressure-assisted sample preparation using different sample types and to investigate the effect on the protein extraction and digestion separately, as it may be the case that PCT is beneficial only at the lysis step. In addition, regardless of the chosen method for protein/peptide identification, an extensive optimization of sample preparation in terms of digestion conditions is crucial to obtain reliable results. It has always to be born in mind that such factors as enzyme to substrate ratio, denaturant, and enzyme storage conditions affect trypsin activity and thus efficiency of the entire process. Meticulous optimization of digestion conditions as well as the choice of the most appropriate strategy for protein/peptide identification are crucial for obtaining the best possible results.

Finally, analysis of FFPE tissue has to consider a high miscleavage rate resulting from extensive crosslinking and frequent lysine residue methylation (+14 Da) introduced by formaldehyde treatment. Therefore, +14 Da modification as characteristic of FFPE tissue should be set in a search engine to diminish this problem. The rate of miscleavage in FFPE tissue samples can be decreased by application of FASP sample preparation method.

Funding sources

The study was funded by project “International Centre for Cancer Vaccine Science” that is carried out within the International Research Agendas Programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund.

BV was supported by the by the European Regional Development Fund - Project ENOCH (No. CZ.02.1.01/0.0/0.0/16_019/0000868) and by the Ministry of Health Development of Research Organization, MH CZ - DRO (MMCI, 00209 805).

Credits

Artur Pirog - Writing **-** Original Draft, Writing - Review & Editing, Visualization, Methodology, Investigation **Jakub Faktor -** Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization **Zuzanna Urban-Wojciuk** - Resources, Investigation **Sachin Kote -** Conceptualization, Methodology, Writing- Reviewing and Editing **Elżbieta Chruściel – Resources, Investigation Łukasz Arcimowicz -** Resources, Visualization **Natalia Marek-Trzonkowska -** Conceptualization, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing **Borek Vojtesek –** Resources **Ted R. Hupp -** Project administration **Sofian Al Shboul –** Resources **Paul M Brennan -** Writing - Review & Editing, Resources **Ryszard Tomasz Smoleński –** Investigation, Writing - Review & Editing **David R. Goodlett** Writing - Review & Editing, Methodology **Irena Dapic -** Conceptualization, Project administration, Writing - Review & Editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The graphical abstract was created with BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122568.

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